OPTIMIZATION OF FERMENTATION PROCESSES

FIELD OF THE INVENTION

[0001] The present invention relates to a method of optimising a bioprocess involving a complex nutrient mixture.

BACKGROUND OF THE INVENTION

[0002] In fermentation processes, *i.e.* conversion of substances by using microorganisms (hereinafter "bioprocesses") complex nutrients are frequently used as an additional nutrient source for microorganisms. Complex nutrients are raw materials containing two or more substances that are necessary for or that promote the growth of microorganisms. Examples of complex nutrients include natural raw materials such as Cornsteep powder or liquor, a waste product in the extraction of starch from maize or yeast extract, and synthetic mixtures of individual substances. A special advantage of these complex nutrients is the wide range of individual substances, such as amino acids, proteins, vitamins, mineral salts or trace elements, which can be made available to the microorganisms. This is an advantage for obtaining high growth rates as compared with the use of minimal chemically defined media.

[0003] There are, however, various problems in using complex natural nutrients in bioprocesses. Since the substances are usually natural, the quality of complex nutrients varies widely in dependence on the manufacturer and the batch. Also the natural composition of complex nutrients is not necessarily optimal with regard to the actual requirement of the microorganisms. Some constituents are present in too small quantities and therefore have a

limiting effect, whereas others are present in excess and are either wasted or even inhibiting. Furthermore the overall metabolism of the many different constituents in complex nutrients is very complicated and partly unknown. Various overlapping adaptation processes may cause violent fluctuations in the process, resulting in irregular productivity and yield from the bioprocess. In multi-stage production processes, this may be a serious problem because the subsequent process steps may be affected. Also non-optimum operating conditions increase costs. Optimization of the medium with regard to a few key substances cannot be regarded as sufficient to remedy these shortcomings because the properties of complex nutrients as a whole are variable, as are the properties of the system of metabolising microorganisms.

[0004] A known means of optimization involves *e.g.* the complete factorial experimental design whereby in a statistical approach is used to evaluate all possible combinations of independent variables under suitable conditions. A model of the system is therefore necessary. Although an optimum is quickly reached in the case of many substances and concentrations under investigation, these methods are impracticable when the number of variables and conditions is larger, owing to the enormous number of experiments required.

[0005] A more efficient optimization strategy is to plan tests to allow for some of the factors by the "Response Surface" method, *e.g.* the Plackett-Burmann method (Greasham and Inamine in: Demain and Solomon (Eds), Manual for industrial microbiology and biotechnology, Washington: ASM 1986, pages 41 – 48) or the Box-Behnken method (Greasham and Herber in: Rhodes and Stanbury (Eds), Applied microbial physiology – a practical approach. Oxford: Oxford University Press 1997, pages 53 – 74). In these methods the number of variables is reduced to those with a significant effect, *e.g.* on growth or product formation.

[0006] Genetic algorithms, in contrast to statistical methods, are non-model-based methods of optimization. With regard to application thereof, this means that they generally

need not be based on theoretical considerations regarding the metabolism of microorganisms. These methods can optimize a large number of media components in a convergent manner. Out of a number of parallel shake flask experiments the best are selected, and the media therefrom become the starting points for the next generation of experiments. The procedure is repeated until convergence is reached. In the first generation the media are varied at random (Weuster-Botz *et al.*, Biotechnol. Prog. 13:387 – 393 (1997)).

[0007] For example, Weuster-Botz *et al.*, Appl. Microbiol. Biotechnol. 46:209–219 (1996) optimized eight trace elements in 180 shake flask experiments by using a genetic algorithm, whereby the L-isoleucine concentration was improved by 50% compared with the standard medium. Weuster-Botz *et al.* (1996) used the same method in an L-lysine process to optimize 13 medium components in 472 standardised shake flask experiments. The L-lysine concentration was improved by over 2% as a result. Compared with statistical formulations including the "Response Surface" method with a conventional complete second-order polynomial model, the number of experiments was appreciably reduced – 472 instead of 2^{13} = 8192; all possible combinations of these parameters would have involved 101^{13} experiments. However, there are serious disadvantages in medium optimization by means of batchwise shake flask experiments. Such disadvantages include the inability to maintain a constant pH, very poor oxygen feed owing to the surface gas admission, and decreased reproducibility due to variation in starter cultures.

[0008] The pulse method in chemostatic culture (Kuhn *et al.*, Eur. J. Appl. Microbiol. 6:341–349 (1979); Goldberg and Er-el, Proc. Biochem. 16:2-81 (1981); Fiechter, Adv. Biochem. Eng. Biotechnol. 30:7-60 (1984); Reiling *et al.*, J. Biotechnol. 2:191-206 (1985)) uses a pulse injection technique to obtain growth reactions on nutrients. This is a means of identifying essential nutrients, the yield coefficients of which can be subsequently determined in a number of chemostatic experiments, in each of which an essential nutrient is the limiting

factor. The yield coefficients can then be used to obtain an optimized-balanced medium. Because the essential nutrients must first be identified, the experimental work is considerable.

SUMMARY OF THE INVENTION

[0009] The optimization methods summarised above are unsatisfactory. Accordingly, one object of the invention is to provide a method for optimising the performance of bioprocesses using complex nutrients, wherein the proportion of complex nutrients in the medium during the process is constantly re-adapted to the actual requirement of the microorganisms and the actual quality of the raw materials.

[0010] One embodiment of the invention is a method of optimizing performance of a bioprocess involving a complex nutrient mixture comprising periodically and alternately stopping a supply of each nutrient in a complex nutrient mixture to a culture of microorganisms until a metabolic activity of the microorganisms decreases by a preset percentage, calculating a new feed concentration of the complex nutrients, and adjusting the amount of each nutrient supplied to the microorganism with an optimization routine.

[0011] Another embodiment of the invention is a device for optimized performance of microbiological processes involving complex nutrient mixtures, wherein a supply of each nutrient is periodically and alternately stopped until a metabolic activity of a microorganism in the process decreases by a preset percentage, whereupon new feed concentrations of the complex nutrients are calculated and adjusted with an optimization routine, the device comprising a reactor for performing the microbiological process with a microorganism comprising at least two individual feed lines for supplying nutrients to the reactor, sensors for measuring a metabolic activity of the microorganism, a co-ordination controller controlled by

the sensors, a multicomponent controller; and elements for controlling the feed concentrations of the complex nutrients.

[0012] A further embodiment of the invention is a method for optimizing production of a fermentation product comprising:

(a) cultivating in a bioreactor a microorganism in a complex nutrient mixture using a first feed concentration;

(b) retarding the flow of a first nutrient from the mixture into the bioreactor;

(c) measuring a metabolic activity of the microorganism and maintaining the retardation of the flow of the first nutrient into the bioreactor until the metabolic activity of the microorganism decreases by a preset value;

(d) calculating a second feed concentration using an optimization routine;

(e) adjusting the first feed concentration to the second feed concentration based on the calculation in step (d); and

(f) repeating steps (a)-(e) until the nutrient mixture supplied to the microorganism is optimized for the production of the fermentation product.

[0013] Another embodiment of the invention is a fermentation system wherein cultivation of a microorganism is optimized for production of a fermentation product, the fermentation system comprising a bioreactor equipped for continuous operation, means for separating nutrients of a complex nutrient mixture into separate streams of the individual nutrients, so that the composition of the mixture that is introduced into the bioreactor may be altered during the fermentation process, means for measuring and controlling pH, pO_2, and temperature in the bioreactor, a device for measuring and controlling the amount of the

nutrient mixture introduced into the bioreactor, means for controlling a feed stream of the nutrient mixture into the bioreactor and for measuring an exhaust-gas composition to provide a gas transfer rate as a measurement signal, and an automation system for controlling the fermentation system.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 is a flow diagram showing one embodiment of the present invention in which an automated laboratory system is depicted. The bottom row shows the four storage bottles (left) and the bottle for caustic soda solution. To the right are the control unit of the bioreactor, the bioreactor itself together with measuring probes and the product container. The lines for gas admission together with the mass flow controller and sterile filter and the CO2 and O_2 analysis for the exhaust gas are represented under the process computer and the serial interfaces. The thin lines indicate the electric wires for data transmission, showing the corresponding form of transmission (RS-232, RS-422 or Mettler Local-CAN).

DETAILED DESCRIPTION OF THE INVENTION

[0015] In the present invention, it has been found that bioprocesses using complex nutrient mixtures can be optimized if the supply of each nutrient is periodically and alternately stopped until the metabolic activity of the micro-organisms decreases by a preset percentage. The time taken on each occasion is used as a response signal whereby new feed concentrations of the complex nutrients are calculated and adjusted by means of an optimization routine. The waiting time between these negative pulses should be between ¼ and 1 hydrodynamic residence time, depending on the dynamics of the process. In some cases, however, the waiting time may be zero or even longer than 5 hydrodynamic residence times. As used herein,

"hydrodynamic residence time" is the ratio of the flow rate (litres per hour) to the reaction volume (in litres).

[0016] In continuous operation of an ideally mixed stirred tank (such as the bioreactors used), theoretically a complete volume exchange in the reactor is never reached. As an approximation, however, in chemical reaction technology, a continuous stirred tank reactor is considered quasi-steady after three hydrodynamic residence times, since it is then calculated that 95% of the volume has been exchanged. In bioprocess technology, however, this time is at least five hydrodynamic residence times, because during the exchange of volume the microorganisms react to the changed environment and, thus, delay reaching a quasi-steady state. In the optimization routine of the invention, there is no need to wait for a quasi-steady state after every negative pulse and this is, therefore, an advantage over conventional pulse-response methods.

[0017] The method according to the invention, as compared with methods based on a positive pulse response wherein the growth rate of the micro-organisms is temporarily increased by a nutrient pulse, also has the advantage that the measured response times are not falsified by lag phases of the micro-organisms. As used herein, a "lag phase" is the time taken by the microorganisms to adapt to changed ambient conditions. It is characterised in that the microbial growth initially remains almost unchanged. In positive nutrient pulses, the measurable reaction to the pulsed nutrient is delayed in a usually non-reproducible manner, thus falsifying the response time.

[0018] The metabolic activity can be measured via observable process parameters, such as the oxygen transfer rate or the carbon dioxide transfer rate. As used herein, "carbon dioxide transfer rate" means the amount of carbon dioxide migrating per unit

time from the liquid phase (fermentation broth) to the gas phase (exhaust gas). It can be directly measured by exhaust-gas analysis. Since the quantity of carbon dioxide not detected by exhaust-gas analysis and leaving the reactor in dissolved form is usually negligible, the rate of formation of carbon dioxide can be equated with the measured carbon dioxide transfer rate for the purposes of the invention. Other parameters of use for controlling the process are *e.g.* the pH, the concentration of dissolved oxygen, and the temperature. The percentage reduction in metabolic activity, measured via the change in the these process parameters, should be chosen at a relatively small value (e.g. 1 – 5%) so that the process is not driven into conditions where the main substrate (*e.g.* sorbitol in the example) is not completely converted.

[0019] In industrial implementation of the optimization process according to the invention, preferably the ratio of the feed concentrations of the complex nutrients and the total quantity of the complex nutrients are regarded as separate control variables but are adjusted simultaneously.

[0020] According to the invention the proportions and the total quantity can be simultaneously adjusted by an optimization routine centred preferably on a multi-component controller. The multi-component controller can *e.g.* be based on fuzzy logic (compare Zadeh, Inf. Control 8:338-353 (1965)). The optimization routine preferably comprises the following three levels:

1. The co-ordination controller for generating the control variables;
2. Multicomponent controllers (*e.g.* fuzzy-logic controllers) and
3. Control of the feed concentrations of the complex nutrients.

[0021] As used herein, "optimization routine" means an arrangement of elements which in co-operation can be used to control the process. An optimization routine according to the invention may *e.g.* involve a co-ordination controller using the negative-pulse response technique, generating response times and using them to form the input variable Q_{sens}. In all cases, the pulse response time is measured for one nutrient while the other is stopped. The reciprocal Q'_{sens} is used as the input variable for the stopped nutrient. The co-ordination controller also calculates the input variable relative to the set value for adjusting the total quantity Ψ_{GM}. The multicomponent controller is run through once for each of the two nutrients ($c_{N1,F,I}$ and $c_{N2,F,I}$ = feed concentrations of the complex nutrients; in this expression the subscripts N1 and N2 denote the various complex nutrients, F denotes the feed concentration and I is a serial subscript within the optimization routine.) The respective feed concentrations of the complex nutrients are then re-calculated via the controller output.

[0022] Control variable for optimising the quantitative proportions:

[0023] The negative pulses are completed for each complex nutrient while the other is stopped. In this case the control variable is the rate of formation of CO_2 (measured as the transfer rate).

[0024] For example, after the supply of one complex nutrient has been stopped, the time is measured before the rate of formation of carbon dioxide decreases by 3%. The optimization algorithm then calculates new feed concentrations for the two complex nutrients. After a fixed waiting time (in this example 5 h corresponding to half the residence time), a negative pulse is completed for the other complex nutrient. Because this method is a convergence method, it is not necessary to reach a steady state after each negative pulse.

[0025] The relevant control variable for optimising the quantitative proportions:

$$Q_{sens} = \frac{\Delta t_i}{\Delta t_{i-1}} \qquad (1)$$

is therefore obtained by dividing the actual pulse response time Δt_i by the pulse response time Δt_{i-1} in the previous cycle, measured with the respective other complex nutrient. In the case of the stopped nutrient, the reciprocal of Q_{sens}

$$Q'_{sens} = \frac{\Delta t_{i-1}}{\Delta t_i} \qquad (2)$$

is used. The same fuzzy-logic controller can therefore then be used, thus appreciably reducing the efforts in tuning the controller. Owing to the hydrodynamic properties of the stirred tank reactor, the response times and consequently the denominators in (1) and (2) cannot become zero.

[0026] The control variable for the total quantity:

[0027] The control variable for the total quantity is the value of a parameter x_{GM} observable during the process and correlated, *e.g.* with the biomass or with the yield of product, *e.g.* the rate of oxygen consumption or the virtual concentration of carbon dioxide. For inputting into the fuzzy-logic controller, the control variable is standardised at the set point, yielding the following general input variable:

$$\Psi_{GM} = \frac{X_{GM}}{X_{GM,soll}} = \frac{\text{control variable}}{\text{set point}} \qquad (3)$$

[0028] Extension to more than two complex nutrients:

[0029] When there are two complex nutrients for optimising, the result is a cycle in two different steps. In principle however the loop can be extended to any number of steps, *i.e.* complex nutrients. In the generalised case of n complex nutrients, the following formula is used:

$$Q_{sens,i} = \frac{(n-1)\,\Delta t_i}{\sum_{q=i-n}^{i-1} \Delta t_q} \qquad (4)$$

[0030] This does not change the parameter Ψ_{GM} for controlling the total quantity. Owing to the long time constants, it is not a practical proposition to optimize more than three or four nutrients.

[0031] Controlling the feed concentration:

[0032] Corresponding to the output x_a of the multicomponent controller, the feed concentrations of the complex nutrients are controlled as follows:

$$c_{k,F,i+1} = x_a \bullet c_{k,F,i}\,,\ k = N1,N2 \qquad (5)$$

[0033] The process of the invention is applicable to all fermentation processes involving complex nutrients, *e.g.* the conversion of D-sorbitol to L-sorbose using a microorganism. The microorganism may be any microorganism useful for the respective conversion, *e.g.* a *Gluconobacter suboxydans* strain may be used for the conversion of D-sorbitol to L-sorbose, *e.g. G. suboxydans* IFO 3291 which was deposited with the Institute for Fermentation, Osaka,

Japan on April 5, 1954, or which was deposited as a mixed culture with *G. oxydans* DSM 4025 under the Budapest Treaty as FERM BP-3813 at the Fermentation Research Institute, Japan, on March 30, 1992.

[0034] For the purpose of continuous cultivation of a microorganism, *e.g. G. suboxydans*, and in order to implement the desired optimization process, the fermentation system comprises

1. a bioreactor equipped for continuous operation;
2. a means for separating the feed of medium into a number of streams of the individual components, so that the composition of the medium can be altered during the process;
3. a means for measurement and control of pH, pO_2, and temperature;
4. a device for measuring and controlling the filling level of the bioreactor to ensure efficient and continuous operation;
5. a means for controlling the feed stream and measuring the exhaust-gas composition, so that corresponding gas transfer rates are available as measurement signals, and
6. an automation system for controlling the bioprocess installation.

[0035] The bioreactor may *e.g.* be a standard laboratory bioreactor with suitable additional equipment and an automation system. Such a bioreactor may include, for example, laboratory system storage bottles and the bottle for caustic soda solution, the control unit of the bioreactor, the bioreactor itself together with measuring probes and the product container, lines for gas admission together with the mass flow controller, sterile filter, the CO_2 and O_2 analysis for the exhaust gas, the process computer and the serial interfaces, and electric wires

for data transmission, the corresponding form of transmission being, e.g. RS-232, RS-422 or Mettler Local-CAN.

[0036] The following examples are provided to further illustrate the methods and devices of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1
Continuous cultivation of *Gluconobacter suboxydans*, wherein D-sorbitol is converted to L-sorbose

[0037] For the fermentation a standard laboratory bioreactor with additional equipment and automation system components according to Figure 1 was used.

[0038] The process computer was a commercial Server-PC. The equipment chosen for the process computer was as follows: Server-PC'Dell PowerEdge 2200'; 2 Intel Pentium II 300 MHz CPUs; 128 MB main memory, 2 graphic cards and 2 screens (21"); 2 Control RocketPort 16 ISA multiport serial cards for a total of 32 serial interfaces, each switchable between RS-232 and RS-422

[0039] **Software:** The operating system of the process computer was Microsoft Windows NT 4.0 (Service Pack 3).

[0040] Automation was based on the industrial software BridgeVIEW, Version 1.1, by National Instruments.

[0041] Fuzzy logic was applied by means of the BridgeVIEW extension DataEngine VI 1.5 by MIT GmbH, Aachen.

[0042] **The Bioreactor:** The bioreactor was a standard Biostat B standard stirred tank reactor with a working volume of 2 litres by B. Braun Biotech International. The inlet air

was introduced into the bioreactor through a silicon flexible tube and a sterile filter (pore size 0.2 µm). The gas introduction means was a gasification ring, also supplied, disposed underneath the 6-blade disc stirrer. The exhaust gas was first conveyed through a condenser on the bioreactor and then through a flexible silicon tube and a sterile filter (pore size 0.2 µm) to the exhaust-gas analysers. The bioreactors were each equipped with a pH electrode and pO_2 probe (both by Ingold) and a temperature probe (PT100). The bioreactors were equipped in the factory with a control unit containing the measurement amplifiers for pH, pO_2, and temperature probes and the initially required standard controllers for these parameters. The transfer of process data and set values between the controller and the process computer occurred via a serial RS-422 interface. The pH electrode was calibrated (two-point calibration at pH = 7.00 and pH = 4.01) before each sterilisation operation. The pO_2 probe was calibrated after sterilisation (single-point calibration with 100% air saturation in the medium).

[0043] Control of filling level of the bioreactor: The filling level of the bioreactor was controlled via the weight. A balance (Mettler Toledo SG32001) was disposed under the reactors and yielded digital signals (serial RS-232 interface) which were converted to a 4-20 mA signal in a digital/analog converter. The analog signal was connected to the input of a hardware controller (Eurotherm), which actuated the discharge pump (Gilson Minipuls 3 peristaltic pump) of the bioreactor, using an analog 0-10 V signal.

[0044] Storage solutions: Five different storage solutions were used to make up the medium, and were each added separately.

[0045] The mass flow measurement was gravimetric. The signals from the balance were transmitted via serial RS-232 interfaces to the process computer. The various balances used are listed in Table 1. LC-RS adapters by Mettler-Toledo were used for the type SG and PG balances.

[0046] To prevent the formation of density gradients, the D-sorbitol storage bottle was stirred by a magnetic stirrer (produced by Variomag) disposed between the balance and the bottle.

[0047] The media were conveyed by peristaltic pumps (Gilson MiniPuls 3) through flexible silicon tubes to the bioreactor. For communication to the peristaltic pumps a serial RS-422 bus was used. To this bus up to ten pumps could be connected. Since the pumps had a so-called GSIOC interface, a suitable adapter was used on each RS-422 bus.

Table 1: Balances used (Mettler-Toledo) and maximum load (ML)

No.	Contents	Type of Balance	ML	Accuracy
1	D-sorbitol	KCC 150s with ID 5	150 kg	1 g
2	Cornsteep	SG32001 DR	32 kg	0.1 g
3	Yeast Extract	SG32001 DR	32 kg	0.1 g
4	Water	SG32001 DR	32 kg	0.1 g
5	Caustic soda solution	PG8002	8 kg	0.01 g

[0048] **Inlet air control:** The inlet air streams were controlled by gas mass flow controllers type 1179 by MKS, Munich, operating on the principle of the hot-wire anemometer. The power supply and analog control and evaluation of the gas mass flow controller were effected via a type 647B 4-channel control device by MKS, connected to the process computer via RS-232. Since the measurements by the controller were based on the thermal capacity of the gas being measured, suitable gas corrective factors were set up. The mass flow of gas was expressed in standard volumes per unit time ($Ncm^3\ min^{-1}$, standard conditions, T = 273.14 K; p = 0.101325 MPa). The measuring range was 2000 $Ncm^3\ min^{-1}$ at

an accuracy of 1.0% of the maximal range. The measured mass flow of gas was calibrated at the factory for nitrogen. For air the gas corrective factor was 1.0.

[0049] **Exhaust-gas analysers:** The exhaust-gas analyser comprised a microprocessor-controlled oxygen analyser OXOR 610 and a microprocessor-controlled NDIR gas analyser (UNOR 610 by Maihak, Hamburg) for measuring carbon dioxide. Both devices were connected to the process computer via RS-232.

[0050] **Sterilisation:** The bioreactor, all the feed and discharge pipes and the vessels for products were sterilised for 20 minutes in a saturated steam atmosphere (0.2 MPa at 121°C). The sterile storage solutions and the air-feed and exhaust-gas lines were connected via special steel sterile couplings.

[0051] **The micro-organism:** The micro-organism *G. suboxydans*, IFO 3291 was used which was deposited under the Budapest Treaty as FERM BP-3813 at the Fermentation Research Institute, Japan on March 30, 1992.

[0052] **Media:** The continuous medium was made up of four separate storage solutions. For simple determination of the dry biomass, all the solutions were free from solids. Because Cornsteep powder contains a high proportion of insoluble constituents, the Cornsteep solutions were suitably processed as set forth in more detail below. The concentrations of the resulting media are given in g/L^{-1}. However, the individual storage solutions from which the resulting medium was made up, were set forth in percentages by weight, to simplify production thereof by weighing in the individual components. The following solutions were used:

1. D-sorbitol solution, 50.4%wt D-sorbitol, $\rho = 1.22$ kg l^{-1}; lot size: 20 l

2. Cornsteep solution: 2%wt Cornsteep powder (Roquette, France) and salts as per Table 2 in demineralized water; batch size: 20 l. Before sterilisation the solution

was centrifuged at 4000 g for 10 minutes. The sterilised solution was filtered into an empty sterile 20 l bottle through a 3 µm deep-bed filter module (Sartorius 5521307P900A, sterile) in front of a 0.2 µm membrane filter module (Gelman Supor DCF CFS92DS, sterile); $\rho = 1.01$ kg l^{-1}.

3. Yeast extract solution; 4%wt yeast extract powder, Oxoid, and salts as per Table 2 in demineralised water; $\rho = 1.01$ kg l^{-1}; batch size: 10 l

4. Water: salts as per Table 2 in demineralised water; $\rho = 1.00$ kg l^{-1}; batch size: 20 l

5. 3 N caustic soda solution for adjusting the pH; $\rho = 1.25$ kg l^{-1}; batch size: 2 l.

[0053] Because the microorganism used always produces small quantities of acid metabolites, no acid was needed for adjusting the pH. The concentrations of the solutions of complex nutrients relate to the corresponding dry powder as weighed in. In the case of the Cornsteep solution, this means that the separated solids are also contained in the stated concentration. Because however solids are not usually bioavailable, however, comparison may be made with Cornsteep solution containing solids as used on the pilot or production scale.

Table 2: Concentrations of salt in the storage solutions 2 to 4 (A) and in the resulting medium (B)

Salt	A at $c_{sit,F}$ = 275 g/l/%	A at $c_{sit,F}$ =137.5 g/l/%	B/(g l^{-1})
$MgCl_2 \bullet 6 H_2O$	0.029	0.021	0.176
KH_2PO_4	0.055	0.039	0.330
$CaCl_2 \bullet 2 H_2O$	0.014	0.010	0.083

[0054] The salt concentration in the resulting medium should be similar to that of synthetic water. The salt concentrations in solutions 2 and 4 and in the medium resulting from the first four solutions can be obtained from Table 2. Since the D-sorbitol solution (solution 1) did not contain any salts, the salt concentrations of solutions 2 to 4 must be correspondingly higher. The consumption of solution 5 was negligible and was disregarded when calculating the salt concentrations.

[0055] All the solutions were sterilised for 20 minutes in a saturated steam atmosphere (0.2 MPa at 121°C).

[0056] A constant dilution rate of D = 0.1 h^{-1} and a constant D-sorbitol concentration of $c_{sit,F}$ = 275 g l^{-1} in the feed were chosen. The feed concentrations of Cornsteep and yeast extract were preset by the optimization process. On each occasion the bioprocess automation system, using the preset concentrations and the dilution rate, calculated the required mass flows for each storage solution. The calculated mass flows were converted and kept constant in the controller incorporated in the bioprocess automation system. The cycle time was 1 second. This ensured that the negative pulses generated by the optimization routine and the newly calculated feed concentrations of the complex nutrients were exactly adhered to.

[0057] Implementation of the optimization routine for this special example: The chosen characteristic measurement signal for the metabolic activity was the carbon dioxide production rate CPR.

[0058] The characteristic measurement signal for the total amount was the virtual carbon dioxide concentration (D-sorbitol equivalent per volume)

$$c_{CO2,virt} = \frac{CPR \cdot M_{Sorbitol}}{6 \cdot 22.4\ l/mol^{-1} \cdot D} \qquad (6)$$

[0059] Because the dilution rate D in general was constant, the variation in the carbon dioxide production rate ("CPR") and in virtual carbon dioxide concentrations ("$C_{CO2.virt}$") vary in linear manner.

[0060] In this example, therefore, the control variable for the total quantity is

$$\Psi_{GM} = \frac{c_{CO_2,virt}}{c_{CO_2,virt,soll}} \qquad (7)$$

calculated from the actual virtual carbon dioxide concentration and the set value for the virtual carbon dioxide concentration.

[0061] In this example the multicomponent controller was constructed in the form of a fuzzy-logic controller with, *e.g.,* the following fuzzy correlation functions: For example, the numerical value 0.7 of the function "very low" is given a correlation of 0.33 and of the function "low" is given a correlation of 0.67. In other words the numerical value $\Psi_{GM} = 0.7$ in the linguistic sense means 33% 'very low' and 67% 'low'.

[0062] The numerical values of the control variables Q_{sens} and Ψ_{GM} or Q'_{sens} and Ψ_{GM} are translated into fuzzy-logic linguistic variables containing so-called correspondence functions. This process is also called 'fuzzyfication'. Since the same fuzzy-logic controller, only with different control variables, was used for the stopped complex nutrient and for the complex

nutrient whose pulse response time was measured during the actual cycle, no distinction hereinafter is made between Q_{sens} and Q'_{sens}. The linguistic input variables Q_{sens} and Ψ_{GM} are linked and the linguistic output variables are associated by 'if.....then' rules. Since the association with the correlation functions is sharp, a number of rules may apply simultaneously.

[0063] The rules are weighted on the basis of the correlation values. The linguistic starting variable is transformed back to a numerical value representing the controller output x_a. This process is also called 'defuzzyfication'.

[0064] Further information about fuzzy logic can be obtained from the relevant literature (Zimmermann (Ed): Fuzzy-Technologien – Prinzipien, Werkzeuge, Potentiale. Düsseldorf: VDI-Verlag (1993)), which is incorporated by reference as if recited in full herein.

[0065] **Fermentation runs completed by using the optimization routine:** The adjusted process was observed over a period of 13 days. The control variable $c_{CO2.virt}$ and both correction variables fluctuate with a time offset of about half a day from one another. The fuzzy-logic controller reacts somewhat too sharply to deviations of the control variable $c_{co2.virt}$ from the set value. These fluctuations, however, do not increase and the process as a whole remains stable. The control variable Q_{sens} fluctuates at irregular intervals around its set value. Even here, no unstable behaviour was observed.

[0066] **Optimising the quantitative proportions:** In order fully to stabilise the control variable Q_{sens}, artificial fluctuations were produced in the quality of the complex nutrients. To this end, the storage bottles of the complex nutrients were respectively replaced by bottles containing complex nutrients from other manufacturers and therefore of different quality.

[0067] A specially informative example was the change form Oxoid yeast extract to Roth yeast extract. The ratio of Cornsteep to yeast extract altered from about 3:1 to 1:1. After

another change back to Oxoid yeast extract, the old proportions were restored. The adjustment took about 3 days or 7 hydrodynamic residence times.

[0068] A change in the yeast extract results, in some cases, in considerable deviations from the set values. The optimization process, however, can keep the process stable even in these situations.

[0069] **Adjustment of the total quantity:** Variation in time of the virtual carbon dioxide concentration $c_{co2.virt}$ and of the feed concentrations of the complex nutrients $c_{CS,F}$ and $c_{YE,F}$ after switching on the optimization process with excursion of the control variable for the total quantity of complex nutrients $c_{co2.virt}$.adjustment of the virtual carbon dioxide concentration $c_{co2.virt}$ from a relatively high starting value of about 13 g l^{-1} to the set value of 6 g l^{-1}. An overshoot is clearly recognisable in the right-hand of part 7. The adjustment took 4 days or about 10 residence times.

[0070] After reaching the set values, the molar yield with regard to L-sorbose improved from 90.3% to 91.1%. The concentrations of complex nutrients $c_{CS,F}$ and $c_{YE,F}$ in the feed were reduced on average by 51% and 56% respectively.

[0071] In accordance with the foregoing the present invention provides

(1) A method of optimizing performance of a bioprocess, wherein e.g. D-sorbitol is converted to L-sorbose, involving a complex nutrient mixture comprising:

(a) periodically and alternately stopping a supply of each nutrient in a complex nutrient mixture preferably comprising two different nutrient mixtures to a culture of microorganisms until a metabolic activity of the microorganisms decreases by a preset percentage;

(b) calculating a new feed concentration of the complex nutrients; and

(c) adjusting the amount of each nutrient supplied to the microorganism, e.g. *Gluconobacter suboxydans*, with an optimization routine preferably comprising a co-ordination controller for

generating control variables, a multicomponent controller, preferably a fuzzy-logic controller, and means for controlling feed concentrations of the complex nutrients.

(2) A method as in (1), wherein the optimization routine comprises:

(a) generating a flow chart with a co-ordination controller using a negative-pulse response technique;

(b) generating response times; and

(c) using the response times to form the input variable Q_{sens}.

(3) A method as in (1), wherein a ratio between the feed concentrations of the complex nutrients and the total quantity of the complex nutrients are treated as separate control variables but are adjusted simultaneously.

(4) A device for optimized performance of microbiological processes involving complex nutrient mixtures, wherein a supply of each nutrient is periodically and alternately stopped until a metabolic activity of a microorganism in the process decreases by a preset percentage, whereupon new feed concentrations of the complex nutrients are calculated and adjusted with an optimization routine, the device comprising

(a) a reactor for performing the microbiological process with a microorganism comprising at least two individual feed lines for supplying nutrients to the reactor;

(b) sensors for measuring a metabolic activity of the microorganism, preferably a pH electrode, or a software sensor like the online calculation of the CPR in the Bioprocess Automation System, preferably being based on the software Natinal Instruments Bridge VIEW Vers. 1.1;

(c) a co-ordination controller controlled by the sensors, preferably a Software Controller as part of the Bioprocess Automation System;

(d) a multicomponent controller, preferably a Software Controller as part of the Bioprocess Automation System; and

(e) elements for controlling the feed concentrations of the complex nutrients, preferably a Software Solution as part of the Bioprocess Automation System with a connected measuring device, e.g. a Mettler balance, and a connected controlling device, e.g. a Gilson MiniPuls 3 peristaltic pump.

(5) A method for optimizing production of a fermentation product comprising:

(a) cultivating in a bioreactor a microorganism in a complex nutrient mixture, preferably comprising e.g. at least two different complex nutrient mixures, using a first feed concentration;

(b) retarding the flow of a first nutrient from the mixture into the bioreactor;

(c) measuring a metabolic activity of the microorganism, e.g. by determining a parameter selected from oxygen transfer rate, carbon dioxide transfer rate, pH, concentration of dissolved oxygen in the bioreactor, and the temperature of the bioreactor, and maintaining the retardation of the flow of the first nutrient into the bioreactor until the metabolic activity of the microorganism decreases by a preset value, e.g. a decrease in the metabolic activity of about 1% to about 5%wt;

(d) calculating a second feed concentration using an optimization routine, e.g. a co-ordination controller for generating control variables, a multicomponent controller, e.g. a fuzzy-logic controller, and a control element for control of flow rate of the nutrients in the complex nutrient mixture into the bioreactor;

adjusting the first feed concentration to the second feed concentration based on the calculation in step (d); and

(e) repeating steps (a)-(e) until the nutrient mixture supplied to the microorganism is optimized for the production of the fermentation product.

(6) A fermentation system wherein cultivation of a microorganism is optimized for production of a fermentation product, the fermentation system comprising:

(a) a bioreactor equipped for continuous operation;

(b) means for separating nutrients of a complex nutrient mixture into separate streams of the individual nutrients, e.g. set-up related in using several bottles, e.g. 4 bottles instead of only one for the feed supply, so that the composition of the mixture that is introduced into the bioreactor may be altered during the fermentation process;

(c) means for measuring and controlling pH, pO_2, and temperature in the bioreactor, corresponding sensors and controllers e.g. intergrated in the bioreactor, e.g. the Braun Biostat B Bioreactor;

(d) a device for measuring and controlling the amount of the nutrient mixture introduced into the bioreactor, e.g. a Software Solution as part of the Bioprocess Automation System with a connected measuring device, e.g. a Mettler balance, and a connected controlling device, e.g. a Gilson MiniPuls 3 peristaltic pump;

(e) means for controlling a feed stream of the nutrient mixture into the bioreactor and for measuring an exhaust-gas composition, e.g. a Software Solution as part of the Bioprocess Automation System with a connected measuring device, e.g. a Mettler balance, and a connected controlling device, e.g. a Gilson MiniPuls 3 peristaltic pump to provide a gas transfer rate as a measurement signal; and

(f) an automation system for controlling the fermentation system.

[0072] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the following claims.